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Monitoring Minimal Residual Disease in Acute Leukemia: Technical Challenges and Interpretive Complexities

Xueyan Chen¹ and Brent L. Wood^{1,2}

¹ Department of Laboratory Medicine, University of Washington, Seattle, WA, USA

² Seattle Cancer Care Alliance, Seattle, WA, USA

Corresponding author:

Brent L. Wood MD, PhD

Professor, Laboratory Medicine and Pathology

Director, Hematopathology Laboratory and SCCA Pathology

Medical Director, SCCA Laboratories

University of Washington, Seattle, WA, USA

Tel.: +1-206-288-7117

FAX: +1-206-288-7127

E-mail: woodbl@u.washington.edu

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Abstract

Minimal residual disease (MRD) after therapy has unequivocal prognostic value in acute leukemia. Over the past 20 years, a number of techniques have evolved into routine laboratory tools to detect MRD, most notably, multiparametric flow cytometry (MFC) and quantitative polymerase chain reaction (PCR)-based molecular methods. There is growing evidence that the presence of MRD detected by MFC or molecular methods provides independent prognostic information and is associated with an increased risk of relapse and shortened survival. However, the predictive value of MRD may be affected by a lack of consensus as to the timing for assessment, the methodology used, and inter-laboratory variation in test performance. Herein, we review the methodological principles of MRD assays, discuss the clinical implications of monitoring MRD for risk stratification and directing further therapy, and suggest potential areas for future investigation.

INTRODUCTION

Acute leukemia including acute lymphocytic leukemia (ALL) and acute myeloid leukemia (AML) is a highly heterogeneous group of diseases with variable response to therapy. Despite intensive chemotherapy, a significant proportion of patients with acute leukemia eventually relapse after achieving initial remission. The source of relapse is the persistence of leukemic blasts at a level below the limit of conventional morphologic detection, that is, minimal residual disease (MRD). In pediatric and adult ALL, MRD has been shown to be highly associated with relapse in many studies [1-17]. Although the data is relatively limited in AML compared to ALL, there is mounting evidence that MRD levels after induction therapy independently correlate with risk of relapse [18-26]. Therefore, given the prognostic value of MRD, it is necessary to develop sensitive, accurate and standardized methods for MRD detection. Over the past decades, various techniques have been developed aiming to detect and monitor MRD more precisely (Table 1). Multiparametric flow cytometry (MFC) and quantitative polymerase chain reaction (PCR) have become powerful tools in MRD detection, and new molecular-based methods, such as high throughput next-generation sequencing (NGS), are evolving to provide even more sensitive measurements. The principles, advantages, and limitations of these methods will be reviewed and the clinical application of MRD assessment will be discussed.

DEFINITION OF REMISSION AND DETERMINATION OF MRD

Following induction therapy, a majority of patients with acute leukemia achieve complete remission (CR) [27,28]. Traditionally, the definition of CR is based on morphologic and clinical criteria; CR requires the presence of <5% blasts in the bone marrow by morphology and peripheral count recovery [29,30]. However, many patients achieving morphologic remission relapse, indicating that morphologic assessment is not adequate to detect the presence of MRD that is responsible for future relapse. The issue is not only one of sensitivity, since in AML the enumeration of blasts by morphology after induction therapy [31] or prior to bone marrow transplant [32] shows little relationship to outcome or to MRD as assessed by flow cytometry, suggesting an additional lack of specificity. These data indicate the need for a revised definition of remission that does not rely on a morphologic blast count, but instead includes more sensitive and specific assays highly correlated with outcome.

Currently, risk stratification is determined by several patient- and disease-related pretreatment factors, such as clinical parameters and cytogenetic risk groups [33]. However, these commonly assessed covariates present at diagnosis have limited predictive value for outcomes, in particular, therapeutic resistance [34]. MRD represents the integration of both the biologic characteristics of leukemic cells and patient-related intrinsic variation including drug metabolism and host response, which together determine a patient's response to therapy. Although inclusion of more pretreatment covariates, such as molecular profiling, may potentially improve prognostication, data gained only after treatment including MRD and peripheral count recovery provide additional independent prognostic information that should be considered when planning future therapy [35]. Highly sensitive methods, e.g., MFC and molecular-based testing, are required to detect MRD for risk stratification, for early recognition of impending relapse, and to direct subsequent therapy. Correspondingly, there is a strong rationale to incorporate MRD status into the criteria for CR to define an immunologic or molecular remission, which would be more predictive of outcome. A standardized description of MRD-based response and monitoring has been established by the Consensus Development Workshop on MRD, including "complete MRD

response”, “MRD persistence”, and “MRD reappearance”, which allows for the comparison of MRD results between different treatment protocols [36].

MRD DETECTION: METHODOLOGIC APPROACHES

Discriminating leukemic blasts from normal hematopoietic precursors is challenging for all methods of MRD evaluation, as there are no clearly defined, universal markers. Several methodologies have been applied to identify residual leukemic blasts based on chromosomal abnormalities, gene deletion/fusion/amplification, aberrant immunophenotype, immunoglobulin/T cell receptor (TCR) gene rearrangements, and molecular mutations [37]. To be clinically informative, MRD assays should be able to consistently detect and quantify residual leukemic cells with high sensitivity and specificity during the course of therapy, and allow implementation with interlaboratory standardization with reproducibility. Currently, MRD is most commonly evaluated by MFC and real time quantitative PCR (qPCR) or reverse transcriptase quantitative PCR (RT-qPCR).

Multiparametric Flow Cytometry

The detection of MRD by flow cytometry relies on the immunophenotypic principle that antigen expression during normal hematopoiesis is highly reproducible between individuals for cells of a given lineage and maturational stage, while leukemic cells show altered patterns of antigen expression reflecting an underlying dysregulated program due to genetic mutation [38]. This fundamental principle is implemented in two related methodological approaches for detecting MRD by flow cytometry.

In the first approach, combinations of antigens expressed by the leukemic blasts that are absent in normal progenitors are identified, each referred to as a “leukemia-associated immunophenotype” (LAIP) [39-41]. At diagnosis, an antibody panel is used to define regions in multiparametric space that contain only leukemic blasts but not normal progenitors. After therapy, the antibody panel found to be informative at diagnosis is used on subsequent samples and leukemic events present in the predefined regions are counted as MRD. An extensive list of LAIPs has been described [23,41-43], including synchronous antigen expression, cross-lineage antigen expression, antigen overexpression/underexpression, and aberrant light scatter. The leukemic blasts at diagnosis may have multiple LAIPs, all of which should be followed in subsequent samples to increase the sensitivity and specificity of MRD detection. With continued improvements in technology, identification of specific LAIPs may be enhanced by adding more fluorochromes, thus allowing more simultaneous markers to define a leukemic blast population with higher confidence. While this strategy has been successfully used in some studies, it has some limitations that affect its clinical utility. First, the immunophenotype of leukemic blasts are not always stable under the influence of therapy or due to leukemic blast heterogeneity [44,45]. It has been reported that immunophenotypic changes in the expression of at least one antigen between diagnosis and relapse were observed in 88-91% of AML patients [46,47] and 98% of patients had a discrete leukemic population present at relapse not detectable at diagnosis [47]. In B-ALL, the use of steroids during induction therapy can reduce the expression of immature antigens and induce the expression of mature antigens [48,49]. Similarly, immature antigens in T-ALL also dramatically decline after therapy [50]. These immunophenotypic and population shifts after therapy can lead to false negative results. Second, similar to the leukemic blasts, the immunophenotype of the background normal or regenerating precursors may also change in

response to therapy and appear in the regions defined as MRD, resulting in false positive results. Lastly, this approach is completely dependent on knowing the diagnostic immunophenotype, without which LAIPs cannot be defined and regions cannot be constructed.

Alternatively, the second approach often termed “difference from normal” identifies leukemic blasts by recognizing immunophenotypic deviation of a population from the patterns of antigenic expression seen on normal hematopoietic progenitors of similar lineage and maturational stage [51,52]. When performed at diagnosis, this is similar to the identification of LAIPs, although it implies an understanding of the maturational sequence for the relevant lineage that is not required to define regions for LAIPs in the first approach. After therapy, the immunophenotypic aberrancies seen at diagnosis may be used as a starting point for evaluation, but all populations are assessed for immunophenotypic deviation relative to the map of normal maturation (Figure 1). This method avoids the limitations of LAIPs caused by immunophenotypic shift, as in a majority of cases it is possible to detect leukemic blasts even after significant immunophenotypic changes. Another advantage over strict LAIP evaluation is that MRD can be assessed without knowledge of the diagnostic immunophenotype, an important factor for reference laboratories or tertiary care centers. This approach also allows use of a standard antibody panel for most cases that includes antigens commonly seen in traditional LAIPs as well as emphasizing normal patterns of maturation [52]. However, this approach does require expert knowledge of antigen expression seen during differentiation of normal hematopoietic progenitors, so training and experience are most important, and the assessment is somewhat subjective making assay standardization and implementation more challenging. In truth, the LAIP approach is largely a subset of the difference from normal approach and components of both methods are commonly used simultaneously in actual practice.

Assay sensitivity is dependent on the degree of immunophenotypic deviation of the leukemic blasts, the number of normal progenitors of similar type, and the number of events acquired in the assay. The number of events recommended for acquisition and the number of events defining a leukemic blast population vary significantly among groups and laboratories, although Poisson counting statistics govern the reproducibility of enumeration. In general, a sensitivity of 0.01% can be achieved in B-ALL and T-ALL and 0.1% in AML for the large majority of patients with higher level of sensitivity being possible for particularly informative immunophenotypes. Assay sensitivity also can vary at different time points post therapy largely due to variable numbers of normal progenitors of similar immunophenotype. For example, assay sensitivity can reach 0.001% at end of induction therapy in B-ALL since the number of normal lymphoid progenitors is very low to absent at this time point, whereas the sensitivity may be significantly reduced at end of consolidation when the normal B lymphoid progenitors are expanded during marrow regeneration. Additionally, specimen type also affects assay sensitivity. In B-ALL, bone marrow is the preferred specimen due to the poor correlation of MRD between bone marrow and peripheral blood, whereas in T-ALL, the MRD levels in paired samples are comparable and strongly correlated [53]. The limited data in AML suggest that MRD levels in bone marrow and peripheral blood are significantly correlated after induction and consolidation therapy, although one log lower on average in the peripheral blood [54]. Currently, the majority of clinical trials perform MRD evaluation on bone marrow samples.

Real Time Quantitative PCR

The development of qPCR assays has provided a major venue to establish standardized methods to assess MRD in a variety of acute leukemia. PCR-based MRD monitoring is only applicable in acute leukemia with detectable mutations or gene rearrangements, which are present in 40-70% of AML and >90% ALL. In ALL, MRD measurement by qPCR primarily relies on analysis of clonally rearranged immunoglobulin or TCR genes. Clonal immunoglobulin heavy chain (IGH) gene rearrangements can be detected in virtually all B-ALL and cross lineage rearrangements of TCR-gamma (TCRG) rearrangements occurs in >50% of patients [55]. The detection rate of PCR targets is lower in T-ALL, although addition of TCR-beta (TCRB) rearrangements as MRD targets increases the sensitivity. For MRD assessment, the specific rearrangements are sequenced at the time of diagnosis and allele-specific oligonucleotide (ASO) primers are designed complimentary to the unique patient or leukemic-specific junctional region sequence [56]. A large-scale standardization has been performed and guidelines proposed for qPCR assays within the Euro-MRD group [57,58]. With additional PCR targets being introduced, 90-95% of ALL patients now have at least two sensitive MRD targets for monitoring [58]. The theoretical sensitivity is 10^{-4} - 10^{-5} (0.01-0.001%).

Common targets for MRD assessment in AML are fusion transcripts from balanced chromosomal translocations detected by RT-qPCR, i.e., PML-RARA, RUNX1-RUNXT1, CBFB-MYH11, and MLL gene fusion, which are present in ~25% of AML in younger patients. The discovery of molecular mutations such as NPM1, FLT3, and CEBPA in fusion gene-negative patients increases to ~60-70 % the AML cases potentially suitable for PCR-based MRD monitoring [59]. RT-qPCR is sensitive (10^{-4} - 10^{-5}) and less complicated to perform since the primer-probe sets targeting the known leukemic transcripts are standard and no patient-specific primers are needed. The Europe Against Cancer (EAC) program established a framework for assessment and standardization of RT-qPCR assays using common primer-probe sets for each respective fusion transcript through systemic parallel evaluation among international expert laboratories [60]. Importantly, the EAC program also identified the most reliable internal control gene, facilitating the comparison of data generated between laboratories. With the implementation of whole genome sequencing and single nucleotide polymorphism arrays, many previously unknown molecular aberrations have been discovered in AML and these findings will expand the pool of potential targets suitable for PCR-based MRD monitoring.

New Molecular Methods

Recently, the application of a Nanoliter-sized droplet technology paired with digital PCR (ddPCR) has allowed the development of more sensitive assays (up to ten fold) than traditional qPCR [61]. This method has potential advantages, including absolute quantification of nucleic acid targets without the use of external reference materials and better tolerance to PCR inhibitors allowing improved amplification efficiency. This approach may improve MRD detection compared with RT-qPCR in chronic myelogenous leukemia [62].

Recently, NGS has demonstrated its potential as a diagnostic platform and has been gradually introduced into clinical practice for MRD detection. The use of this technique for B and T-ALL MRD monitoring relies on the high-level multiplexing capability of NGS to allow the design of families of balanced primers that simultaneously amplify all possible combinations of the rearranged IGH or TCR loci. This eliminates the need for patient-specific primers and allows creation of a general purpose MRD assay for nearly all patients. This approach has been applied

to T-ALL, in which NGS for TCRB and TCRG identified clonal TCR rearrangements at diagnosis and at day 29 post therapy for MRD assessment [63] in the majority of patients. The patients where NGS was uninformative were T-ALL of immature immunophenotype where rearrangement of TCR had not yet occurred. Similarly, NGS for IGH genes performed in B-ALL allowed detection of clonal IGH rearrangements at diagnosis in 90-95% of patients and successfully identified MRD in day 29 post-therapy samples [64]. In both diseases, the NGS results were highly concordant with flow cytometric MRD detection, but also identified a significant subset of patients where MRD was detected by NGS at a very low level below the limit of detection of flow cytometry. While this suggests that NGS MRD detection is more sensitive than flow cytometry, the clinical significance of this finding is unclear, particularly since the B-ALL cohort was a pediatric low/standard risk population in which relapse was less frequent. Recently, it has been demonstrated that MRD detection by NGS can reproduce risk stratification similar to that seen using PCR, suggesting NGS likely can replace PCR in clinical trial designs [65]. In AML, the feasibility of using NGS for the detection of NPM1-mutated AML has been demonstrated using a bar-coded small molecule error correction approach (smMIPS). Salipante et al. showed the sensitivity of NPM1 detection by NGS reached 0.001%, comparable to qPCR [66]. Furthermore, NGS could detect MRD in a subset of flow cytometry-negative cases and detect alternate NPM1 mutations in addition to the index mutation in one third of patients. The growing evidence strongly supports the technical feasibility and potential clinical utility of NGS for MRD monitoring in acute leukemia. However, similar to the LAIP approach for MRD detection by flow cytometry, access to diagnostic material is obligatory to obtain an index sequence for IGH and TCR MRD detection, a potentially significant limitation.

CLINICALLY RELEVANT SENSITIVITY AND TIMING OF MRD DETECTION

Many studies have unanimously shown that MRD detected after induction therapy is a strong prognostic factor for outcome in pediatric and adult ALL, both in newly diagnosed and relapsed leukemia, as well as in patients undergoing allogeneic hematopoietic cell transplant (HCT) [36,67]. As a result of these studies, virtually all pediatric ALL patients are being monitored for MRD to assess treatment response and direct future therapy. While the presence of MRD following therapy is associated with a poor prognosis, it is challenging to incorporate into clinical practice given that MRD is a continuous variable and most studies have dichotomized MRD for risk stratification with variable cut-off values used in different trials. From a clinical perspective, it is important to recognize that the relevant level of sensitivity for risk stratification and the timing of assessment are closely related to the therapeutic protocol used and the rate of response, so direct extrapolation of the results from one treatment regimen to another must be done with caution.

Acute Lymphoblastic Leukemia

Prognostic Significance of MRD

The prognostic significance of MRD quantification was first established in pediatric ALL. The first large scale prospective study by van Dongen et al. monitored patients with childhood ALL and assessed the prognostic values of MRD, measured at nine different time points in the first 3 months after treatment using semi-quantitative PCR analysis of IG kappa and TCR genes [68]. Different levels of MRD ($\geq 10^{-2}$, 10^{-3} , $\leq 10^{-4}$) had independent prognostic value, especially at the first two time points. At early time points, a higher level of MRD ($\geq 10^{-2}$) correlated with a higher relapse rate when compared with patients with a lower level of MRD ($\leq 10^{-4}$), whereas at

later times, even a low level of MRD was associated with poor outcome. The adverse prognostic significance of MRD at the end of induction for event-free survival (EFS), disease free survival (DFS) and overall survival (OS) has been confirmed by additional prospective pediatric studies using either qPCR or flow cytometry sensitive to a level of 10^{-4} [2, 8, [69]. In particular, day 29 marrow MRD has been identified as the most important prognostic variable for outcome [2]. Levels of MRD between 10^{-4} and 10^{-5} by flow cytometry at end of induction in high-risk patients are also associated with a small reduction in EFS [6] in comparison to those where no MRD is detected. Further from therapy, the small subset of patients with detectable MRD at $>0.01\%$ at the end of consolidation therapy has been shown to have a particularly poor outcome [2], as does persistent MRD prior to HCT [69]. Earlier in therapy, the clearance of blasts by day 8 from blood is associated with improved EFS and identifies a subset of patients with good outcome [2]. Finally, end of induction MRD is not simply a surrogate for good risk cytogenetics, as MRD has been shown to be strongly prognostic and correlated with a worse outcome in patients with favorable cytogenetic features [2].

To investigate whether MRD detected by high sensitivity NGS better correlates with outcome, Pulsipher et al. reported in B-ALL patients enrolled in COG trial ASCT0431 that MRD detected by NGS predicted relapse and survival more accurately than 6-color flow cytometry-based assessment in both pre- and post-HCT settings [70]. Absence of detectable MRD by NGS pre-HCT identified low-risk patients potentially eligible for less intense therapy. Any levels of post-HCT MRD detected by NGS were highly predictive of relapse and survival, especially early (day 30) after HCT, suggesting the utility of NGS to identify patients requiring early post-HCT intervention.

In T-ALL, only few trials with MRD assessment based on a relatively small number of patients have been published [71-73]. The AIEOP-BFM-ALL 2000 study reported the first large series of children with T-ALL and prospectively evaluated MRD at two time points using qPCR [74]. MRD negativity at day 33 post induction was the most favorable prognostic factor and MRD $\geq 10^{-3}$ at day 78 was the most important predictive factor for relapse.

In adult ALL, studies from different groups have demonstrated the strong independent prognostic impact of MRD post induction and during early consolidation therapy, using qPCR [11,75-80] or flow cytometry [16]. Depending on the various time points assessed and the patient population, each study group used its own MRD cut-off values for risk stratification. The Programa Español de Tratamientos en Hematología (PETHEMA) ALL-AR-03 trial showed the presence of MRD $\geq 10^{-3}$ after induction (weeks 5-6) and $\geq 5 \times 10^{-4}$ after early consolidation (weeks 16-18) by flow cytometry were the only prognostic factors for DFS and OS in Ph-negative high-risk ALL [16]. Bassan et al. reported persistent MRD $\geq 10^{-4}$ by RT-qPCR for fusion transcripts at weeks 16-22 correlated with a lower 5-year DFS/OS rate in adult ALL patients treated on protocol Northern Italy Leukemia Group (NILG)-ALL 09/00 [76]. Using a more sensitive method, MRD detected by NGS within 30 days before HCT conditioning predicted post-HCT relapse in a small cohort of adult B-ALL patients [81]. The presence of MRD ($\geq 10^{-6}$) in blood samples before day 100 post-HCT had 100% positive predictive value for relapse.

To explore the correlation between flow cytometry and qPCR for MRD assessment and their capacity to predict outcome, a Swedish multi-center study analyzed the parallel use of these two methods in pediatric ALL treated according to Nordic Society of Paediatric Haematology and Oncology (NOPHO) ALL-2000 protocol [82]. Using a MRD threshold of 0.1%, the overall concordance between qPCR of IG/TCR and flow cytometry was high in detecting MRD at day 29 in B-ALL and T-ALL. qPCR in general showed higher MRD levels than flow cytometry, perhaps related to differences in specimen processing or denominator effects. In B-ALL, MRD detected by both methods predicted a higher risk of relapse using a cut-off of $\geq 0.1\%$ at day 29, whereas in T-ALL, day 29 MRD detected by qPCR was superior compared to flow cytometry in predicting relapse.

The evidence supporting the independent prognostic significance of MRD in B-ALL is compelling despite assessment at various time points using different techniques and thresholds, suggesting that it is a robust marker. However, the cut-off for clinically relevant MRD levels is highly dependent on the methods used for determination, the time points for monitoring, the intensity of treatment regimen, and the anticipated outcomes. Therefore, MRD thresholds for decision making need to be defined within each individual trial and cannot be directly compared between trials.

Role of MRD in Risk-Directed Therapy

Because of the significant prognostic value of MRD, the utility of MRD in risk stratification and guiding further therapy are being explored in clinical trials of ALL (Table 2). MRD may be used to identify high-risk populations that benefit from more intensive therapy. In COG's high-risk B-ALL study AALL0232, intensified therapy given to MRD-positive patients at the end of induction ($>0.1\%$) did not improve survival, although the early relapse rate was similar to that seen in MRD-negative patients, suggesting intensified therapy altered the disease course in MRD-positive patients and transiently improved outcome. Vora et al. also reported that in high risk patients (MRD $\geq 10^{-4}$ at the end of induction) randomized to receive standard or augmented post-remission therapy, 5-year EFS and OS (numerically) was better in the augmented treatment group than in the standard group (89.6% vs. 82.8% and 92.9% vs. 88.9%, respectively) [83], suggesting that MRD high risk patients may benefit from augmented therapy. Similarly, the assignment of patients to even more intensive therapy (HCT) based on the presence of MRD has been demonstrated to improve EFS, OS and cumulative incidence of subsequent relapse (CIR) in both pediatric [84] and adult ALL [16, 75, 76, 78, 79].

In contrast, MRD may be useful to identify low-risk populations that may benefit from less intense therapy in an effort to reduce toxicity. For example, children and young adults with MRD low risk ALL (undetectable or $< 10^{-4}$ by qPCR at the end of induction) treated on the Medical Research Council UK ALL 2003 (MRC UKALL 2003) trial were randomized to receive one or two delayed intensification courses [85]. No significant difference in EFS was observed between the two groups receiving one or two delayed intensification, implying that treatment reduction is feasible for low risk patients defined by MRD levels at the end of induction. In addition, it has been demonstrated that HCT does not appear to be beneficial for patients with very low ($\sim < 10^{-4}$) or absent MRD [16, 78].

These studies confirm the importance of MRD in ALL risk assignment and suggest MRD stratification help identify subgroups of patients for risk-directed interventions that may improve outcome.

Acute Myeloid Leukemia

Multiparametric flow cytometry MRD assessment

Although data are relatively limited, multiple studies have demonstrated a similar correlation between the presence of MRD after therapy and poorer prognosis in pediatric and adult AML [21,23-25,86-88]. While monitoring MRD by flow cytometry has become standard practice in pediatric and most of adult ALL for risk assessment and therapeutic decision making, this technique has not been universally applied to risk stratification in AML. This is in part due to the greater difficulty in assessing MRD in AML secondary to the heterogeneity of the disease at both an immunophenotypic and genetic level. The clinically relevant cut-off for MRD positivity assessed by flow cytometry in published trials varies with therapy intensity, treatment schedule, and time point of monitoring. Studies from AML Cooperative Group (AMLCG) demonstrated early detection of MRD by flow cytometry during aplasia following induction therapy was an independent prognostic factor for shorter relapse free survival (RFS) [86]. A cut-off of 0.15% was selected to define MRD-positivity during aplasia and a cutoff of 0.3% for post induction. Interestingly, there was no significant correlation between MRD positivity during aplasia and post induction. Al-Mawali et al. also defined a threshold of 0.15% as an optimal value to discriminate MRD-negative from MRD-positive and demonstrated post induction MRD independently predicted RFS and OS [89]. Late time point MRD assessment after consolidation has been proposed by others to provide more relevant prognostic information. Several studies indicate that the presence of MRD ($\geq 0.035\%$) after consolidation is associated with a higher risk of relapse and shorter RFS and OS [18,25,90]. Early time point MRD assessment may be useful to identify high risk patients for upfront intensive therapy or HCT; however, of patients with slow blast clearance, approximately 30%, who are MRD-positive after induction therapy become MRD-negative after consolidation [18,90]. Therefore, continuous monitoring with serial sampling might be a consideration in certain circumstances. In AML patients who received HCT, the presence of any level of MRD by flow cytometry in CR1 or CR2 prior to HCT was associated with increased risk of relapse and death for both myeloablative and nonmyeloablative procedures [91-93]. Furthermore, the outcomes of MRD-positive patients were similar to each regardless of the levels of MRD ($> 1\%$, $> 0.1\%-1\%$, $\leq 0.1\%$), but worse than that of MRD-negative patients. The combined assessment of MRD pre-transplant and at day 28 post-transplant confirmed that pre-transplant MRD was associated with relapse, but day 28 MRD did not impact risk assessment aside from a very small population of MRD negative patients at transplant that become MRD positive at day 28 and who had a particularly poor outcome [94]. Combined MRD negativity at both time points was highly correlated with a good outcome.

In pediatric AML, Inaba et al. reported the presence of MRD ($\geq 0.1\%$) after induction 1 or 2 by flow cytometry predicted lower EFS and higher relapse rate [31]. In one of the first multi-center MRD-guided clinical trials, AML02, MRD $\geq 1\%$ after induction 1 was the only independent prognostic factor for EFS and OS [88]. Using targeted therapy or HCT based on MRD levels improved clinical outcome in childhood AML. In patients treated on COG AML protocol AAML03P1, the presence of MRD ($\geq 1\%$) after induction 1, 2 or consolidation therapy was

associated with shorter RFS and OS, and was most predictive in relapse in patient without cytogenetic or molecular risk factors [87].

As seen in ALL, the threshold and optimal timing for MRD assessment in AML vary in different patient populations, which is likely correlated with the rate and degree of response to therapy due to underlying patterns of genetic mutation. This is confirmed by RT-qPCR studies in that AML with different leukemic fusion transcripts demonstrate strikingly different kinetics of relapse, suggesting the clinically relevant time points for measurement and the sampling interval are determined by the type of genetic abnormalities [95]. Although considered a different concept from MRD evaluation, kinetics of response as manifested by clearance of blasts reflects the chemosensitivity of the blasts to induction therapy and is associated with outcome in ALL [2]. In AML, early blasts clearance from peripheral blood assessed by flow cytometry during induction therapy also predicts CR and is correlated with higher RFS and OS [96-99].

Molecular MRD Assessment

The utility of RT-qPCR to monitor MRD was best established in acute promyelocytic leukemia (APL), which provides a model for risk directed management in other types of molecularly defined AML. In the Medical Research Council (MRC) AML15 trial, sequential monitoring of PML-RARA transcripts (10^{-4}) proved to be the strongest independent predictor for RFS and has been used to dictate pre-emptive therapy to reduce overt relapse [100]. The persistence of PML-RARA transcripts after consolidation therapy or recurrence of PCR positivity during remission was highly predictive of overt hematologic relapse [101,102]. However, given the current survival rates exceeding 80% among patients treated in clinical trials with successful retreatment at relapse, there has been debate regarding the relevance of routine sequential monitoring for *PML-RARA* transcripts beyond post consolidation time points [103]. In patients with standard risk disease, subsequent sequential MRD monitoring appeared to offer only limited benefit and should be avoided in this group. However, a subset of patients with high risk disease may still benefit from sequential MRD monitoring, which allows early intervention in molecular relapse. Additionally, MRD assessment is still essential to guide treatment of relapsed APL [104].

RT-qPCR assays for core-binding factor (CBF)-positive AML have also been investigated in several clinical trials. The presence of CBF-fusion transcripts has been shown to provide independent prognostic information and assessment at specific time points can identify patients with high relapse risk [20,26,105,106]. A multi-center prospective CBF-2006 trial by the Acute Leukemia French Association (ALFA) and Groupe Ouest-Est des Leucémies et Autres Maladies du Sang (GOELAMS) identified that a less than 3-log reduction in RUNX1-RUNX1T1 or CBFB-MYH11 leukemic transcripts after first consolidation was the sole prognostic factor for a higher rate of relapse, whereas KIT and/or FLT3-ITD/TKD gene mutations showed poor discrimination of high- vs. low-risk patients [20]. Furthermore, Zhu et al. reported that the failure to achieve a 3-log reduction in RUNX1- RUNX1T1 transcripts at the end of second consolidation, but not after induction or first consolidation, was significantly correlated with high relapse risk and that HCT in patients developing early molecular relapse could improve outcome [106]. A prospective study of patients with CBF leukemia treated in the United Kingdom Medical Research Council (MRC) AML-15 trial also defined clinically relevant threshold transcript levels and showed rising MRD levels on sequential monitoring predicted hematologic relapse [26]. Therefore in CBL-leukemia, a combination of monitoring the level of leukemic

transcript at defined time points and sequential monitoring of MRD kinetics provide an accurate prediction for outcome and should be considered in risk-stratification for future therapy.

In AML harboring other translocations, several studies have shown that RT-qPCR analysis can be used to detect MRD in AML with t(9;11) MLL-AF9 [107], t(6;11) MLL-AF6 [108], and t(6;9) DEK-CAN [109]. While the clinical significance of MRD by RT-qPCR needs to be confirmed in a larger cohort, the current data demonstrate that RT-qPCR provides a sensitive tool to monitor response in these patients and the MRD status may be useful to make treatment decisions.

In concordance with the findings in APL and CBF leukemia, MRD assessment by qPCR can identify patients with high risk of relapse in NPM1-mutated AML [22,110,111]. Kronke et al. revealed that the presence of higher NPM1 mutant transcript levels after double induction and consolidation therapy significantly correlated with a higher incidence of relapse and death [22]. Serial MRD monitoring led to early detection of relapse suggesting > 2% NPM mutant/ABL1 may be important to trigger early intervention. The study by Shayegi et al. also emphasized the potential value of serial MRD monitoring to predict relapse, reporting an increase of >1% NPM1mutant/ABL1 was most prognostic after chemotherapy, whereas an increase of >10% NPM1mutant/ABL1 was most prognostic after HCT [111]. As seen in CBF leukemia, MRD detected at later rather than early time points appeared to be more prognostically relevant in NPM1-mutated AML. Wermke et al. reported treatment of a patient with NPM1 molecular relapse who did not develop subsequent overt hematologic relapse and went on to receive a HCT [112], suggesting the possibility of using MRD to drive pre-emptive therapy of molecular relapse as a bridge to HCT. Most recently, Ivey et al. demonstrated that peripheral blood measurement of NPM1 transcripts at the end of second induction is highly correlated with relapse risk and outcome as an independent prognostic factor [113], and remains informative in patients having concurrent FLT3 or DNMT3A mutation. While the data to date has been exclusively generated by qPCR, the feasibility of using NGS for MRD detection in NPM1+ AML has recently been demonstrated [66] and offers the possibility of both high-sensitivity and detection of multiple simultaneous molecular targets through multiplexing. Given that the kinetics of relapse are strikingly different among molecular subgroups including NPM1, PML-RARA, RUNX1-RUNXT1, and CBFB-MYH11 AML [114], the optimal timing for monitoring these molecular signatures is also likely to vary markedly and clinical trials are necessary to define clinically relevant monitoring strategies.

In contrast to AML with specific molecular lesions, early assessment of Wilms tumor gene (WT1), which is not leukemia specific but over-expressed in most acute leukemia, can improve risk stratification in AML [115]. Cilloni et al. reported greater than 2-log reduction in WT1 transcripts after induction predicted reduced risk of relapse and provided independent prognostic information even after adjusting for other co-variants. Additionally, WT1 expression post HCT was the strongest predictor of relapse and could be used as a marker to guide early intervention, such as donor lymphocyte infusion [116]. Recent studies indicate that the impact of WT1 expression on prognosis correlates well with MRD detected by flow cytometry; log clearance of WT1 (lower than 1.96) post induction and MRD by flow cytometry ($\geq 0.2\%$) post consolidation similarly predicted relapse [117,118]. However, given the relatively limited sensitivity and lack of specificity of *WT1*, this assay appears less likely to be implemented into routine practice.

An increasing number of recurrently mutated genes have been discovered in AML, suggesting their use as targets for MRD monitoring. RUNX1 mutations are pathologic molecular aberrations in AML and have been proposed as a clinically relevant biomarker to follow disease progression from myelodysplastic syndrome to AML [119] and are highly stable during the disease course [120]. Kohlmann et al. revealed that residual leukemic blasts with RUNX1 mutations could be detected by NGS with high sensitivity and patients reaching MRD negativity had a better outcome than those with detectable MRD [121]. Separation of patients into “good responder” and “bad responder” based on median residual mutation burden (3.6% threshold) detected post therapy (median day 128) revealed significant differences in EFS and OS. Therefore, RUNX1 qualifies as a marker for MRD monitoring and NGS-based MRD assessment may improve risk stratification. However, it is necessary to validate this finding in randomized clinical trials prior to recommending that RUNX1-mutated patients with MRD should receive intensive therapy and/or HCT.

OPTIMAL METHODS FOR MRD ASSESSMENT

In the United States, MRD evaluation by flow cytometry is rapidly becoming the standard of care in ALL and the most commonly employed method in AML. The major advantages of flow cytometry are general applicability, assay availability, relative affordability, and rapid turnaround time, the latter facilitating prompt decision-making for therapeutic intervention. It also allows simultaneous evaluation of expression levels of multiple antigens on the leukemic blasts, which are relevant for potential targeted immunotherapy. In contrast to the limited genetic targets available in AML for PCR-based assay, > 95% of patients demonstrate an aberrant immunophenotype that can be detected by flow cytometry. Instead of using patient- or mutation-specific primers by PCR, a standard antibody panel for each type of acute leukemia can be applied essentially in all patients with a sensitivity of 0.1-0.01%. The main challenge is considerable variability in the equipment, reagents, data analysis methods, and reporting used in MRD evaluation, resulting in a lack of reproducibility [122,123]. Additionally, flow cytometry has the disadvantage of requiring a high level of expertise for data interpretation that is inherently subjective. Consequently, the development of a standardized assay with demonstration of interlaboratory comparability is necessary to allow comparison of results between different laboratories and different treatment protocols.

In contrast, MRD by PCR-based assays is extensively optimized and standardized. qPCR analysis of IG/TCR rearrangement in ALL is one log more sensitive (0.01-0.001%) in general than flow cytometry, but more laborious, time-consuming, and expensive. In AML, despite the high sensitivity of quantitative PCR-based methods to monitor fusion transcripts and molecular mutations, several concerns have been raised. First, MRD levels cannot be accurately enumerated as the number of transcripts per leukemic cell may vary between patients and be altered after chemotherapy. Second, the genetic mutations detected at diagnosis may not be present at relapse, which affect their utility as MRD markers. For example, instability of FLT3-ITD has been documented in ~20-30% of paired diagnostic and relapsed samples [124-126], and NPM1 mutations become undetectable at relapse in 9% of cases [22]. Thirdly, mutant transcripts may persist in the differentiated leukemic blasts that undergo therapy-induced differentiation, even though, their leukemogenic potential is lost [127,128].

Although flow cytometry and PCR do not generate entirely comparable results, most of the studies showed a concordance rate >90%, if a cut-off of 0.01% is used, in both ALL [26,82,129-133] and AML [134]. The discordance between flow cytometry and PCR, mostly flow-negative/PCR-positive, can be explained by higher sensitivity of PCR assay, non-specific amplification of normal DNA, dead blasts detected by PCR but not flow cytometry, presence of regenerating blasts interfering the identification of leukemic blasts by flow cytometry, and immunophenotypic changes post therapy. Discrepancies are more frequently observed in cases with low levels of MRD ($<10^{-4}$), whereas in samples with high levels of MRD, the results are generally concordant between both methods [135]. In B-ALL, it has been suggested that flow/PCR discordant cases have a clinical outcome intermediate between concordantly positive and negative cases [130]. While MRD by flow cytometry is an independent prognostic factor in CBL leukemia, monitoring RUNX1-RUNX1T1 or CBFB-MYH11 fusion transcripts has been shown to be uninformative in childhood AML and did not improve prediction [31]. A plausible explanation would be the persistence of leukemic transcripts in differentiating blasts after therapy, which lack leukemogenic potential. Therefore, caution should be exercised when using these assays to assess treatment response and direct therapy, particularly at early time points.

NGS has demonstrated higher rates of positivity in post-treatment samples from patients with B-ALL [64], T-ALL [63] and AML [62]. However, it is not clear that higher frequency of positivity at early time points after therapy translates to improvements in risk stratification, so caution is warranted until larger clinical trials with outcome data are available. In the flow cytometric evaluation of post treatment samples, regenerative blasts may be present with an immunophenotype overlapping that of leukemic blasts, resulting in over- or under-estimation of residual leukemic burden. NGS is not subject to these technical difficulties and is likely to ultimately provide more accurate estimation of disease burden than flow cytometry, particularly at later time points. However, the use of NGS clonality assays for MRD assessment in ALL requires access to diagnostic material to obtain an index sequence, which is not required by flow cytometry, and the turn-around time for results is still substantially slower for NGS. Compared to quantitative PCR for leukemia-associated mutations, NGS eliminates the need for mutation-specific probes or knowledge of the prior mutation subtype, e.g. NGS is applicable to all NPM1-mutated AML in regardless of the specific mutation subtype. Because of these apparent advantages, NGS will likely soon be applied to monitor MRD for other informative genes, such as DNMT3A, RUNX1, IDH1/2, etc. [136-138] in a multiplexed assay. This new approach for MRD monitoring has the potential for improved assay standardization and may eventually replace other MRD methodologies. However, several issues still need to be addressed, including higher cost, slower turn-around time, and need for error correction strategies when PCR amplification is used.

PRACTICAL ISSUES AND FUTURE PERSPECTIVES

Discordant MRD Outcomes

MRD is an independent prognostic factor in acute leukemia. However, in a subset of patients, the clinical outcome is different from that predicted by MRD. Up to 25% of patients without detectable MRD after induction therapy eventually relapse, indicating leukemic blasts are likely still present in these patients at a level below the limit of detection by current techniques or were not present in the sample evaluated (false negatives). With increasingly sensitive MRD assays, e.g. expanding antibody panels for flow cytometry, automated flow cytometry data analysis

[139,140], integration of additional MRD markers, and implementation of new technologies, the number of MRD negative patients is likely to decrease at all time points. On the other hand, a subset of patients with MRD positivity have a long RFS, and MRD levels below a certain threshold do not correlate with relapse [26,114,141], something likely to become even more evident as higher sensitivity MRD assays are employed. To date, MRD measurements have largely been made at relatively early time points after therapy and prior to the completion of therapy, so possible explanations for these false positives include damage to residual leukemic blasts that lead to delayed cell death, leukemic blasts that have lost leukemogenic potential through therapeutic selection or differentiation, or further reductions in residual disease from the remaining therapy to be delivered. There may also be host mechanisms such as effective immune surveillance that keep low levels of residual disease in check. Regardless, it is unrealistic to expect that MRD, particularly when measured at an early time point and regardless of assay sensitivity, will correlate perfectly with either outcome or relapse. Instead, measurement of MRD at early time points allows one to define thresholds that balance false negatives (MRD negative that relapse) and false positives (MRD positive that do not relapse) to obtain cohorts of patients with differing overall outcomes suitable for assessment of response to therapies in clinical trials. It is more difficult to apply MRD to individual patients as an assay for relapse assessment, rather it should be considered a prognostic or risk factor.

The prediction is that application of more sensitive MRD assays at early time points will result in a decrease in clinical false negatives at the expense of increasing clinical false positives. The practical result will be identification of a more uniform but smaller population having good outcome at the expense of including more truly good outcome patients having clinically irrelevant low MRD positivity along with the truly poor outcome patients. However, it may be useful to define a very good risk population in this way when reduction in therapy is contemplated. The corollary is that further from therapy, particularly following completion of therapy, the identification of any level of MRD is likely to be more strongly associated with poor outcome, so more sensitive MRD assays may be more clinically relevant at later time points. The tradeoff is an increased rate of false negatives due to MRD being below the assay limit of detection secondary to prolonged intensive therapy.

MRD Monitoring During CR

There is no consensus regarding the appropriate interval for MRD monitoring in patients no longer receiving therapy. It is not clear how long it will take MRD to progress to overt hematologic relapse and how often MRD testing should be performed to ensure detection and allow intervention. Given that different subtypes of acute leukemia have different relapse kinetics [114], the schedule may vary significantly. Future studies are needed to support the rationale of continuous MRD monitoring and pre-emptive therapy, and to investigate the optimal targets and methods for monitoring in CR.

Estimation of Disease Burden

The whole body disease burden post therapy is likely the most relevant factor for predicting outcome, but is difficult to reliably estimate, particularly since MRD is calculated based on the leukemic involvement of a small bone marrow or peripheral blood sample. Regardless of what MRD assay is used, the relatively limited number of cells being analyzed and the possible heterogeneous distribution of the disease may yield an inaccurate estimate of disease burden, the

extreme case being false-negative results. Indeed, positron emission tomography (PET) scanning of marrow reveals marked spatial heterogeneity during and after therapy, indicating response to therapy also varies considerably throughout the marrow [142]. The combination of heterogeneity of leukemic involvement and unrepresentative sampling might contribute to the unexpectedly poor outcome in apparently MRD-negative patients.

Heterogeneity of Leukemic Blasts

Leukemic blasts are heterogeneous and may contain multiple genetic and immunophenotypic subclones at the time of diagnosis or relapse [143-145], so models presuming uniform response by leukemic blasts are overly simplistic and unlikely to be correct. For instance, a leukemic subclone present at the time of diagnosis may gain additional genetic mutations to become responsible for relapse; alternatively, a minor subclone at presentation may be selected by chemotherapy and expand as a chemotherapy-resistant relapse. In an attempt to explain the role of mutations in leukemic heterogeneity and ontogeny, Lindsey et al. divided AML mutations into de novo AML related (e.g., NPM1, CBL and MLL rearrangements), pan-AML (e.g., RUNX1, FLT3, TET2), and MDS related secondary (e.g., SRSF2, U2AF1, ASXL1) mutations [146]. De novo AML related mutations are regarded as leukemogenic, whereas pan-AML mutations can be both primary and secondary events and may be lost or gained when subclones outgrow. Additionally, a few pre-leukemic genetic mutations have been described, such as DNMT3A mutation that persists at remission and where the mutant allele burden does not correlate with clinical course in AML [147]. DNMT3A mutations, along with TET2 and ASXL1, likely represent a marker for age-related clonal hematopoiesis of indeterminate potential [148-150], a potential pre-leukemic state. Consequently, markers for MRD detection should ideally target driver mutations that are required for maintenance of the leukemic state, e.g. de novo AML related mutations, or immunophenotypic aberrations in the early leukemic stem cell population that contain the self-renewal properties of the leukemia. This presumes a better understanding of leukemia biology than is currently the case, so assay for driver mutations is just beginning to be utilized in routine clinical practice. Given that current MRD assays rely largely on features of the bulk blast population at diagnosis, it is not surprising that current MRD assays are not able to recapitulate the risk of relapse. Novel methods are needed, in particular, techniques that allow direct observation of leukemic heterogeneity at the single cell level, including leukemic stem cell populations, in order to improve both our understanding of treatment response and assessment of relapse risk.

A General Strategy for MRD Detection

It has been proposed that MRD monitoring include both early assessment of response to therapy to improve risk stratification and to direct post-remission therapy, and post-treatment monitoring to detect early relapse and guide pre-emptive intervention [151]. Early assessment requires assays with rapid turn-around time in order for the results to be used in real time clinical decision-making, such assays are flow cytometry and PCR. These assays need only be of moderate sensitivity, since application of a threshold appears to be required to optimize clinical significance. The one exception where higher sensitivity at early time points may be relevant is the small subpopulation of patients having rapid and excellent response to therapy in whom reduced intensity therapy might be an option. Assessment of MRD late in therapy or after completion of therapy is likely to benefit from highly sensitive assays in order to reduce the clinical false negative rate, PCR and more probably NGS are likely to play a major role here.

Thus, a general strategy for MRD detection likely will require use of more than one technology, flow cytometry or PCR MRD assessment at an early time point, e.g. end of induction, and PCR or more probably NGS at a later time point, e.g. end of therapy or beyond.

CONCLUSION

MRD detection using current sensitive laboratory techniques has provided significant prognostic value in acute leukemia. Future approaches will require more uniform, disease- and treatment-specific protocols that include a schedule for MRD monitoring using appropriate targets, standard procedures, and clinically validated thresholds that are applied in all laboratories. This approach will ensure optimal and consistent results for risk assessment and personalized therapy. Finally, prospective studies using standardized protocols in randomized trials are necessary to prove the efficacy of MRD-directed therapeutic interventions.

PRACTICE POINTS

- MRD after therapy provides significant value in prognosis and risk-directed therapy in acute leukemia
- MRD monitoring includes both early assessment of response to therapy and post-treatment monitoring for relapse
- Optimal methods for MRD detection depend on the characteristics of the acute leukemia and the clinical scenario
- Clinically relevant sensitivity and timing of MRD detection are closely related to the therapeutic protocol and the rate of response

RESEARCH AGENDA

- Develop standardized flow cytometry assays and new molecular methods to improve reproducibility, sensitivity and specificity of MRD detection
- Determine the optimal schedule for MRD monitoring
- Evaluate the importance of continuous MRD monitoring and pre-emptive therapeutic intervention
- Define optimal targets and methods for monitoring MRD in CR

Conflict of Interest Statement

All authors declare that they have no conflict of interest.

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Figure Legend

Figure 1. The detection of MRD for acute leukemia by flow cytometry. Bone marrow following induction therapy was analyzed with an informative antibody combination. The combination allows the recognition of normal hematopoietic precursors and identifies residual leukemic blasts by deviation from their closest normal counterparts based on lineage and maturational stage.

A. AML MRD. The leukemic blasts (orange) that represent MRD are characterized by abnormal expression of CD33 (increased), CD34 (increased), CD38 (decreased), HLA-DR (variably decreased), and CD56 relative to normal CD34-positive progenitors (red). The population is enumerated at 0.07% of the white cells.

B. B-ALL MRD. The leukemic blasts (red) that represent MRD are characterized by abnormal expression of CD10 (increased), CD34 (increased), CD38 (decreased), and CD58 (increased) relative to most immature normal B cell precursors (cyan). The population is enumerated at 0.03% of the white cells.

C. T-ALL MRD. The leukemic blasts (red) that represent MRD are characterized by abnormal expression of CD3 (absent on the surface, present in the cytoplasm), CD5 (decreased to absent), CD7 (increased), and CD48 (absent) relative to mature T cells (green). The population is enumerated at 0.11% of the white cells.

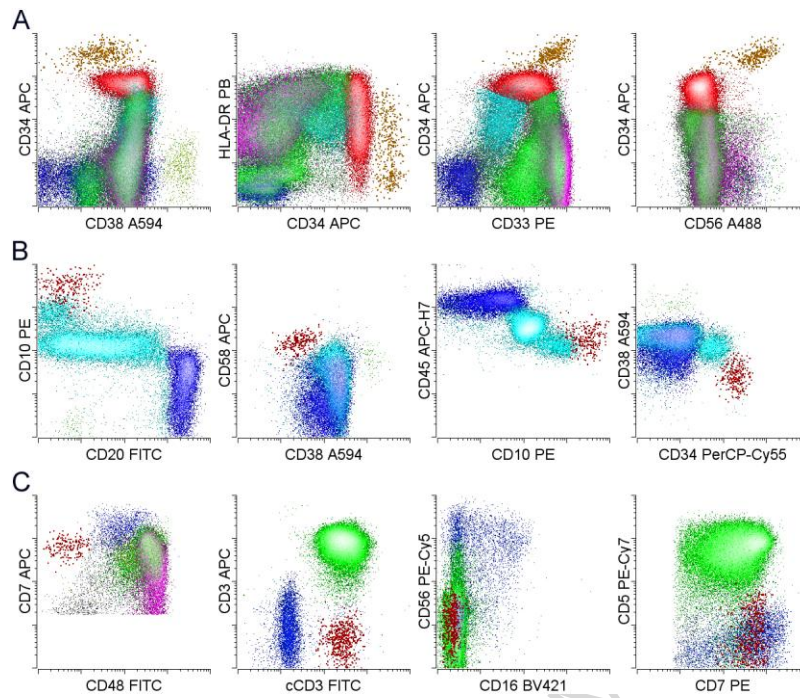


Figure 1

Table 1. Methodologies of MRD detection.

	Chromosome analysis	FISH	MFC	qPCR	RT-qPCR	Next Generation Sequencing	
Sensitivity	1-5%	0.3-5%	3-4 colors: 0.1-0.01%	0.01-0.001%	0.01-0.001%	0.0001%	
			6-10 colors: 0.01-0.001%				
Specimen requirement	Fresh viable cells	Fresh or paraffin embedded tissue	Fresh viable cells	DNA	RNA	DNA	
Turn-around time	4-7 days	2 days	1-2 days	~ 4 weeks to generate primers	1-3 days	1-2 weeks	
Target	Numerical or structural chromosome abnormalities	Gene deletion, amplification, or fusion	"difference from normal" or leukemia-associated immunophenotype	IG/TCR gene rearrangements	Leukemic fusion transcripts	Mutated genes	
Example	t(8;21)	PML-RARA, RUNX1-RUNX1T1	Cross lineage antigen expression	Junctional regions of IGH, IGK, TCRB, TCRG	PML-RARA	Junctional regions of IGH, IGK, TCRB, TCRG; NPM1	
Applicability	Subset of acute leukemia	Subset of acute leukemia	All acute leukemia	Majority of lymphoblastic leukemia	Subset of acute leukemia	Most acute leukemia	
Cost	Less expensive	Less expensive	Moderate expense	More expensive	Moderate expense	Most expensive	
Availability	Widely available	Widely available	Widely available	Widely available in Europe	Widely available	Largely experimental	

Advantages	Standard technique	Rapid	Rapid	High sensitivity	Rapid	High sensitivity	
	Direct quantification	Standard technique	Direct quantification	Highly standardized analysis	High sensitivity	Generally applicable	
		Direct quantification	Generally applicable		Highly standardized analysis	Readily standardized	
			Potential to detect cells with leukemic stem cell phenotype			Can detect new mutations	
						Do not need patient-specific primers	
Disadvantages	Limited sensitivity	Limited sensitivity	Inadequate standardization among laboratories	High cost	Only applicable to acute leukemia harboring detectable abnormalities	High cost	
	Require metaphase cells	Only applicable to acute leukemia harboring detectable abnormalities	Requires high-level of expertise to interpret data	Time consuming		Time-consuming	
	Laborious		May miss blasts with immunophenotypic shifts	Laborious	Product may be in more mature cells that lack leukemic potential	Requires prior knowledge of mutation status for IG/TCR gene rearrangements	
			May be confounded by regenerating blasts	Requires prior knowledge of mutation status for IG/TCR gene rearrangements			
				Uncertain quantification of			

				leukemic blasts			
				May miss blasts with clonal evolution			

FISH, fluorescent in situ hybridization; MFC, multiparametric flow cytometry; PCR, polymerase chain reaction; qPCR, real time quantitative PCR ; RT-qPCR, reverse transcriptase quantitative PCR.

Table 2. Use of MRD in risk-directed therapy in ALL patients.

Refer ence	ALL	Study	Study populatio n	MRD Detection			MRD-directed therapy (No. of patients)	Outcom e
				Method	Timing	Cut off		
Boro witz ⁶	Pediatric B-ALL, high-risk	AALL0232	2473	Flow cytomet ry	End of induction (day 29)	0.0 1%	Intensified therapy to patients with MRD>0.1% (404)	Did not improve 5-year EFS or OS. Early relapse rate similar to that seen in MRD- negativ e patient.
Eckert ⁸⁴	Pediatric ALL, intermediate-risk	ALL-REZ BFM 2002	236	qPCR	End of induction (week 5)	10 ⁻³	HCT (103)	Patients with MRD ≥10 ⁻³ who receiv ed HCT had improve d probabil

								ity of EFS from 18% ± 7% to 64% ± 5% and reduced CIR from 59% ± 9% to 27% ± 5%.
Vora ⁸⁵	Children and young adult Ph-negative ALL, low-risk	MRC UKALL 2003	3207	qPCR	End of induction (day 29) and recovery from consolidation	10^{-4}	Patients with MRD low-risk ($<10^{-4}$) received one or two delayed intensification courses (521)	No significant difference in EFS between the groups receiving one or two delayed intensification.

Vora ⁸³	Children and young adult Ph-negative ALL, standard-risk and high-risk	MRC UKALL 2003	3207	qPCR	End of induction (day 29) and recovery from consolidation	10^{-4}	Patients with MRD high-risk ($\geq 10^{-4}$) received standard or augmented post-remission therapy (533)	5-year EFS was better in the augmented group than in the standard group (89.6% vs. 82.8%). OS at 5 years was numerically higher in the augmented group than in the standard group (92.9% vs. 88.9%).
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Gokbuget ⁷⁹	Adult Ph-negative ALL, standard-risk and high-risk	GMALL	1648	qPCR	day 71 and week 16	10^{-4}	HCT (108)	Patients with MRD $\geq 10^{-4}$ who received HCT had longer probability of continuous CR after 5 years than those without HCT (66% $\pm$ 7% vs. 12% $\pm$ 5%).
Dhedin ⁷⁸	Adult Ph-negative ALL, standard-risk and high-risk	GRAALL	955	qPCR	6 weeks after induction initiation	10^{-3}	HCT (282)	Patients with MRD $\geq 10^{-3}$ had longer RFS. Patients

								with MRD < 10^{-3} did not benefit from HCT.
Bassan ^{75,76}	Adult ALL	NILG-ALL 09/00	280	qPCR	week 16 , week 22	10^{-4}	HCT (59)	Patients with MRD $\geq 10^{-4}$ who received HCT had improved 6-year DFS (42% vs. 18%). Post-transplant outcome was affected by post induction MRD level.

Ribera ¹⁶	Aolescents and adult Ph-negative ALL, high-risk	PETHEMA ALL-AR-03	326	Flow cytometry	End of induction (weeks 5 to 6) and end of the third consolidation cycle (weeks 16 to 18)	5×10^{-4}	HCT (71)	Patients with MRD $\geq 5 \times 10^{-4}$ who received HCT had 5-year EFS and OS probability of 24% and 31%, respectively.
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ALL, acute lymphoblastic leukemia/lymphoma; CIR, cumulative incidence of subsequent relapse; DFS, disease-free survival; EFS, event-free survival; HCT, hematopoietic cell transplant; OS, overall survival; Ph, Philadelphia chromosome; qPCR, real time quantitative PCR; RFS, relapse-free survival.